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REGULATION AND EXPRESSION OF LCR PLASMID-MEDIATED PEPTIDES IN PESTICINOGENIC *YERSINIA PESTIS*

By

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ABSTRACT

REGULATION AND EXPRESSION OF LCR PLASMID-MEDIATED PEPTIDES IN PESTICINOGENIC *YERSINIA PESTIS*

By

Allen K. Sample

Yersinia pestis, the causative agent of bubonic plague, undergoes an ordered metabolic step-down of growth when cultivated *in vitro* at 37°C in Ca^{2+}-deficient medium. Growth restriction is dependent on carriage of an approximate 72 kilobase low calcium response plasmid (Lcr^+) and results in expression of plasmid-encoded functions including V antigen. The Lcr plasmid also encodes a set of novel yersiniae outer membrane proteins (Yops) which are expressed by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, but not by *Y. pestis*, at 37°C in Ca^{2+}-deficient medium. *Yersinia pestis* also possesses a unique 10 kilobase pesticin plasmid (Pst^+). It is shown in this thesis that cells of Lcr^+,Pst^- *Y. pestis* KIM are able to express Yops at levels comparable to that of Lcr^+ *Yersinia pseudotuberculosis*. Pulse-chase radiolabeling with ^{35}S-methionine was used to demonstrate that Lcr^+,Pst^+ *Y. pestis* synthesized at least 11 distinct peptides during the low calcium response and that seven of the labeled peptides were rapidly degraded. These seven peptides were stably expressed in Lcr^+,Pst^- *Y. pestis* and were of identical molecular weights as the Yops expressed by that

strain. Radiolabeled fragments of low molecular weight accumulated in the extracellular medium of Pst^+ cultures and were assumed to be stable degradation fragments derived from Yops. It was also shown that the set of stable peptides, including V antigen, were made during restriction by both Pst^+ and Pst^- Y. pestis KIM and were located primarily within the cytoplasm. Those radiolabeled peptides which underwent proteolytic degradation in Pst^+ Y. pestis were localized to the outer membrane and extracellular medium in the Pst^- strain. It is concluded that the failure of Lcr^+,Pst^+ Y. pestis to express Yops is the result of post-translational degradation and is not a block in the synthesis of Yops.

Dedicated to my parents and my wife, Dawn, who always believed in who I was and what I wanted to be. Thanks to all my friends at Michigan State who kept me from going sane during my five years in the basement of Giltner Hall. Special thanks to Dudley Xavier Sample for supplying me with Tuna Dacquiris and making my house a bar.

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LITERATURE REVIEW

Yersinia pestis, the causative agent of bubonic plague, is a gram-negative bacterium capable of growth and multiplication within mammalian phagocytic cells (27). Y. pestis is an extremely virulent and invasive organism; less than ten organisms injected subcutaneously in a mouse will cause a fatal infection (88). The plague bacillus was first isolated and described by Alexander Yersin in 1894 during a plague epidemic of Hong Kong (96). The organism was assigned to the genus Pasteurella until 1970 when it was placed in the newly-formed genus Yersinia.

Plague is usually transmitted by fleas which ingest plague bacilli while feeding on an infected animal and regurgitate the bacteria during subsequent blood meals on uninfected hosts. After gaining access to a susceptible host, the bacteria rapidly disseminate from the site of the flea bite and enter the dermal lymphatic system. They localize in the lymph nodes (especially the inguinal lymph nodes) and multiply, causing severe pain and swelling. Eventually the bacteria overwhelm the capacity of the lymphatic system and enter the circulatory system, liver, lungs, and spleen. Once the disease becomes systemic, death follows within a few days unless antibiotic therapy is initiated. If Y. pestis localizes in the lungs it causes pneumonic plague with a copious, bloody sputum; coughing by the victim creates an aerosol of the

pathogen. Inhalation of these airborne bacteria results in rapid development of pulmonary disease which is uniformly fatal if not treated promptly.

In addition to Y. pestis, there are two other species of yersiniae which are pathogenic for man, Yersinia enterocolitica and Yersinia pseudotuberculosis. Both of these species are capable of causing enteric disease in humans and gain access to the host via ingestion of contaminated water or food. This route of infection obviates the need for the highly invasive properties characteristic of Y. pestis; both of the enteropathogenic species have a substantially reduced virulence for mice compared to Y. pestis, particularly via peripheral routes (See Table 1).

The yersiniae, Y. pestis in particular, have proven to be instrumental in the elucidation of pathogenic mechanisms of microorganisms. The property of a pathogen's invasive functions being genetically linked to bacteriocin production was first described in Y. pestis (17). The ability of many pathogens to acquire iron via siderophore-independent mechanisms and to utilize exogenous hemin as an iron source was initially reported for Y. pestis (51) as was the importance of iron privation as a non-specific mechanism of host defense (52).

Yersinia pestis also has served as an important model for the pathogenesis of facultative intracellular parasites. It possesses several distinct and well-defined virulence factors (see Table 2); the loss of any one factor imparts some degree of avirulence to the organism. The extreme lethality of the plague bacillus allows studies

TABLE 1. The LD_{50} of wildtype yersiniae for mice by various routes of infections.[a]

Yersinia species	Route of infection[b] s.c.	i.p.	i.v.
Y. pestis	<10	<10	<10
Y. pseudotuberculosis	1.6×10^5	2.4×10^4	2.0×10^1
Y. enterocolitica	2.3×10^3	2.1×10^2	1.0×10^2

a. Data taken from Une and Brubaker (88)

b. Abbreviations are as follows:
s.c., subcutaneous injection
i.p., intraperitoneal injection
i.v., intravenous injection

TABLE 2. Virulence factors of *Yersinia pestis* and their effect on the LD_{50} for mice via various routes of infection.[a]

Phenotype of *Y. pestis*[b]					Route of infection[c]		
Pur	Fra	Pgm	Pst	Lcr	s.c.	i.p.	i.v.
+	+	+	+	+	$<10^1$	$<10^1$	$<10^1$
-	+	+	+	+	$>10^8$	$>10^8$	$>10^8$
+	-	+	+	+	NR[d]	$<10^1$	NR
+	+	-	+	+	$>10^7$	$>10^7$	1.5×10^1
+	+	+	-	+	$>10^7$	10^5	7.1×10^1
+	+	+	+	-	$>10^7$	$>10^7$	$>10^7$

a. Data taken from Brubaker (12), Brubaker, Beesley, and Surgalla (13) and Une and Brubaker (88).

b. Abbreviations used are as follows:
   Pur, purine biosynthesis
   Fra, expression of Fraction 1 capsular material
   Pgm, the ability to absorb exogenous hemin
   Pst, carriage of the pesticin plasmid
   Lcr, carriage of the low calcium response plasmid

c. Abbreviations used are as follows:
   s.c., subcutaneous injection
   i.p., intraperitoneal injection
   i.v., intravenous injection

d. NR stands for not reported in the literature.

of virulence to be performed on the basis of simple mortalities ofinfected animals rather than measurements of survival or proliferation of the organisms *in vivo*. The use of avirulent or conditionally-virulent strains of *Y. pestis* permits the organism to be handled safely in the laboratory for physiological or genetic studies. The individual virulence determinants of *Y. pestis* are described in detail below.

PURINE BIOSYNTHESIS

The ability to synthesize purines (Pur^+) is an inherent requirement of any pathogen and is not a virulence factor unique to *Yersinia pestis*. Mutations to purine auxotrophy resulting in avirulence have been reported for *Salmonella typhosa* (1), *Klebsiella pneumoniae* (39), *Pseudomonas pseudomallei* (59), and *Bacillus anthracis* (50), as well as *Yersinia pestis* (18). It is presumed that free purines are unavailable within the mammalian host and that pathogens must be able to synthesize purines *de novo* in order to initiate and maintain an infection (1). This premise is supported by the *in vitro* infection of cultivated macrophage with *Yersinia pestis*. Although Pur^+ strains were able to grow within macrophages in the absence of exogenous purines, purine auxotrophs were unable to survive or replicate intracellularly unless hypoxanthine and guanosine were added to the culture medium (84).

Metabolic blocks in *Y. pestis* which prevent the synthesis of inosine monophosphate (IMP), an early step in purine biosynthesis,

result in a slight reduction of pathogenicity for mice. In contrast, lesions which block the conversion IMP to guanosine monophosphate result in the complete loss of virulence (11). Mutations which block the conversion of IMP to adenine monophosphate have not been described in Y. pestis, nor have purine auxotrophs of the enteropathogenic yersiniae been reported.

FRACTION 1

Fraction 1 (Fra^+) is a highly immunogenic capsular antigen produced by Y. pestis (77). It has been purified and described as a protein-carbohydrate complex (2) which was maximally produced at 37°C, although small amounts were detected from cells cultivated at lower temperatures (36). Mutation to Fra^- has been reported to occur at a relatively high frequency both in vivo and in vitro (19,70). A second class of mutations, designated $F1^{\pm}$ by Burrows (20), produce fraction 1 antigen but fail to incorporate the antigen into an extracellular capsular structure.

The precise role of fraction 1 in pathogenicity has not yet been determined. Fra^- strains of Y. pestis were of reduced virulence for guinea pigs via intraperitoneal injection (19), but remained fully virulent for mice when injected intraperitoneally (12). Burrows and Bacon were unable to demonstrate any effect of visible encapsulation of Y. pestis on resistance to ingestion by murine phagocytes in vivo (24). Fra^- strains were also reported to be able to cause chronic and

sometimes lethal infections in laboratory rats (92,93). The isolation of $F1^{\pm}$ Y. pestis from a plague patient has been described (94) and makes suspect the importance of fraction 1 antigen in the pathogenicity of Y. pestis for humans.

PIGMENTATION

The ability of Y. pestis to absorb exogenous hemin and form pigmented colonies (Pgm^{+}) on semi-solid medium containing hemin was initially reported by Jackson and Burrows (51). Surgalla and Beesley (85) extended this observation to include absorption of the planar dye Congo red to form pigmented colonies (85). Nonpigmented isolates were demonstrated to be avirulent via subcutaneous or intraperitoneal injection (52,88) but were fully virulent when administered intravenously (88). This conditional pathogenicity makes Pgm^{-} strains ideal for studying the other virulence determinants of Y. pestis safely in the laboratory although in a strain which can still be tested for virulence in the mouse model (88).

Although the mutation rate for Y. pestis from Pgm^{+} to Pgm^{-} was reported to be relatively high, $\sim 10^{-5}$ mutations per generation (10), back mutations to Pgm^{+} have not been detected (51). This high mutation rate and apparent irreversibility is highly suggestive of an extrachromosomal element controlling the pigmentation phenotype, but so far no plasmid has been detected which is correlated with pigmentation (34,87). The exact nature and location of the gene(s) conferring the

pigmentation phenotype have not yet been established, but it is generally assumed that pigmentation is a chromosomally-encoded function (79).

Jackson and Burrows (52) showed that injection of sufficient iron to saturate serum transferrin of the host allows Pgm$^-$ strains to cause a lethal infection via the peripheral routes. This was taken to be evidence that pigmentation plays a role in iron acquisition within the mammalian host. Siderophores were not produced by any of the yersiniae when cultivated under iron-limiting conditions and only the enteropathogenic yersiniae were capable of utilizing exogenous siderophores (68). This suggested to the authors that the yersiniae rely on a cell-bound high-affinity iron uptake system to obtain iron in an iron-deficient environment. Nonpigmented strains of Y. pestis have been shown to be unable to grow for more than a few generations in iron-deficient medium whereas pigmented strains continued to grow for at least 16 generations (79). Whether this reflects an inability to store iron or is a lesion in an iron uptake system is not clear.

The enteropathogenic yersiniae were found to be nonpigmented on the Congo red agar described by Surgalla and Beesley (85), but clinical isolates of Yersinia enterocolitica were reported to bind Congo red when cultivated on the medium of Prpic et al (75). This pigmentation phenotype was correlated with the presence of the Lcr plasmid and was limited to virulent isolates of Y. enterocolitica (75,76). These observations have not yet been expanded to include isolates of Yersinia pseudotuberculosis. Virulent strains of Escherichia coli, Shigella species and Neisseria meningitidis have also been reported to exhibit

pigmentation on a modified Congo red agar (67) which suggests a common role for the pigmentation phenotype in pathogenicity.

PESTICIN PLASMID

Ben-Gurion and Hertman first described an extracellular activity from Yersinia pestis which inhibited the growth of Yersinia pseudotuberculosis, but did not inhibit any other enteric organisms tested (5). The inhibitory activity was expressed by all but one of the twenty-four Y. pestis strains which were examined. They concluded that the inhibitory agent was a protein-like substance on the basis of its inactivation by heat and by proteolytic enzymes. Expression of the activity was induced by ultraviolet radiation and could not be detected in non-irradiated culture supernatants. They termed the activity "pesticin" to denote its similarities to the previously described bacteriocins; colicins from Escherichia coli, pyocins from Pseudomonas aeruginosa and megacin from Bacillus megaterium.

Brubaker and Surgalla reported that pesticin activity was inhibited by the presence of iron or hemin and was enhanced by the presence of calcium ions or chelating agents (14). Using a medium with added calcium chloride and EDTA, they were able to detect pesticin within the cytoplasm of Y. pestis and demonstrate that pesticin was also produced by non-irradiated cultures (15).

Isolates of Yersinia pestis that produce pesticin also produce an inhibitor of pesticin (immunity protein) in order to prevent self-

destruction (14). Strains of Y. pestis which are Pst⁻ fail to produce the immunity protein and may be either sensitive or resistant to the effects of exogenous pesticin. Brubaker reported that of the thirteen nonpesticinogenic strains of Y. pestis tested, all three pesticin-sensitive strains were pigmented and all but one of ten resistant strains were nonpigmented. Using a pesticin-sensitive, pigmented strain of Y. pestis, fifty pesticin-resistant mutants were selected and all were found to be nonpigmented. Conversely, the selection of fifty nonpigmented mutants from the same strain were all found to be resistant to pesticin. It was concluded that pesticin and hemin may absorb to the same site on sensitive strains of Y. pestis (10).

Other bacterial species inhibited by pesticin are limited to the genus Yersinia and to certain strains of Escherichia coli. E. coli ϕ, a commonly used colicin indicator strain (32,37), was reported to be sensitive to the effects of pesticin, while E. coli K-12 was naturally resistant to pesticin (14). Some serotype O:8, O:21 and O:4,32 isolates of Yersinia enterocolitica (32,69) and serotype Ia and Ib isolates of Yersinia pseudotuberculosis were sensitive to pesticin (25) while other serotypes have not been demonstrated to be inhibited by pesticin. Une and Brubaker (88) reported pesticin-resistant mutants of enteropathogenic yersiniae, in a manner similar to Pgm⁻ Y. pestis, were of reduced virulence via peripheral routes. Unlike nonpigmented Y. pestis, pesticin resistant mutants were shown not to have lesions in iron uptake, but to have lost the ability to invade HeLa cells in vitro (79).

Pesticin has been purified to homogeneity as judged by sodium

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (49). It was characterized as single polypeptide of 65 kilodaltons as estimated by sedimentation equilibrium and gel filtration or 44 kilodaltons as judged by SDS-PAGE. Two active isomers designated α-pesticin and β-pesticin were isolated with isoelectric points of 5.49 and 5.87 respectively. Both forms were rapidly converted to a biologically inactive form upon incubation at room temperature (48).

Purified pesticin was used to demonstrate that pesticin possesses N-acetylglucosaminidase activity (33). Pesticin inhibited the *in vivo* incorporation of ^{14}C-labeled diaminopimelic acid into the peptidoglycan of *Escherichia coli* ϕ and promoted both the *in vivo* and *in vitro* release of diaminopimelic acid from peptidoglycan. Pesticin was capable of cleaving peptidoglycan prepared from both pesticin sensitive and resistant strains as well as several different classes of peptidoglycans indicating that resistance to pesticin resides at the level of absorption and not substrate specificity.

In addition to pesticin production, most strains of *Yersinia pestis* possess coagulase and fibrinolysin activities (53,61). Coagulase is responsible for coagulation of plasma and fibrinolysin is involved in the dissolution of clots. It has been assumed that both factors activate existing components in the plasma (prothrombin and plasminogen) which in turn mediate the clotting of the plasma and subsequent lysis of the clot (4). Pesticin, coagulase and fibrinolysin activities were reported to be absolutely correlated (17,31); selection of mutants which lacked one determinant invariably lacked all three activities (21).

Although Beesley *et al* physically separated pesticin activity from

both coagulase and fibrinolysin, the latter activities could not be differentiated on the basis of physical characterisitics (4). Coagulase and fibrinolysin were found primarily in the particulate fraction of disrupted cells. Both showed the same kinetics of heat-inactivation when boiled in Tris buffer; approximately 50% of the activities remained after 30 minutes. Fibrinolysin activity was eluted from a Sephadex G-200SF gel filtration column near the void volume indicating a large apparent molecular weight; coagulase activity could not be detected in any fractions presumably because of dilution during chromatography. Straley and Brubaker identified two distinct outer membrane proteins from Pst^+ Y. pestis which were absent from isogenic Pst^- cells (83). Neither protein was of the same molecular weight previously reported for pesticin and therefore they concluded that the proteins represented the coagulase and fibrinolysin activities.

Failure to separate the ability to produce pesticin, coagulase and fibrinolysin led Brubaker et al to postulate that all three activities are genetically linked and are controlled by an episomal factor (17). It was later shown that an approximate 10 kilobase plasmid is associated with pesticinogeny (6,34); loss of this plasmid resulted in the concomitant loss of pesticin, coagulase and fibrinolysin activities.

Strains of Y. pestis which lack the pesticin plasmid showed a marked reduction in virulence, particularly via peripheral routes, indicating that the pesticin plasmid may be responsible for the invasiveness of the plague bacilli (13,86). The LD_{50} for mice of a wildtype strain of Yersinia pestis was shown to be less than ten organisms by intravenous, intraperitoneal, or subcutaneous injections.

In contrast, a nonpesticinogenic strain was shown to have an LD_{50} of 71 organisms when injected intravenously, an LD_{50} of $3.8x10^5$ organisms via intraperitoneal injection and was completely avirulent when injected subcutaneously. Virulence could be restored to Pst$^-$ Y. pestis administered intraperitoneally, but not subcutaneously, if the mice were also injected with 40 μg of ferrous ion (13). This effect of iron on virulence is similar to that previously reported for non-pigmented strains of Y. pestis (52) and may reflect suppression of non-specific host defense mechanisms rather than an inability of nonpesticinogenic strains to obtain iron in vivo (79,89,90).

LCR PLASMID

Early research concerning the nutrition and physiology of virulent strains of Yersinia pestis was impeded by a rapid population shift of cultures to avirulence. The loss of virulence primarily occurred when cultures were grown at 37°C in standard bacteriological media (38). Wessman et al (91) showed that virulent cells cultured in a defined medium at 37°C failed to replicate and eventually underwent autolysis. Autolysis could be prevented by the additon of 22 mM Mg^{2+}, but the organisms were still unable to replicate. Avirulent cells were able to grow normally at 37°C and eventually predominated in cultures after extended incubation; no differences were observed in growth rates or viability between virulent and avirulent cells when cultivated at 26°C. Ogg et al (65) reported that the in vitro shift to avirulence could be

avoided by the addition of sterile spent culture filtrates, increasing the pH to 7.8 or higher, or reducing the oxygen tension; all of the treatments allowed virulent cells to replicate at 37°C and compete with avirulent cells. They also showed that cells grown in cellophane sacs surgically placed within the peritoneal cavity of a guinea pig retained virulence.

It was demonstrated by Higuchi et al (46) that the population shift to avirulence at 37°C could also be prevented by the addition of 2-4 mM calcium. Under these conditions virulent cells did not undergo lysis and were able to grow equally well as avirulent cells. The addition of zinc or strontium cations were also shown to allow growth of virulent cells. The severity of restriction has been reported to be a function of Mg^{2+} levels; decreased amounts of Mg^{2+} reduced the concentration of Ca^{2+} needed to permit vegetative growth (16). Using a medium which mimics mammalian intracellular fluid with respect to divalent cations (20 mM Mg^{2+}, 0 mM Ca^{2+}), Zahorchak and Brubaker (98) showed that restriction could be relaxed but not prevented by the addition of exogenous adenine triphosphate (ATP). ATP was not metabolized by Y. pestis suggesting that ATP served as a chelator of Mg^{2+} effectively reducing the concentration of free Mg^{2+} and the stringency of restriction.

A shift in incubation temperature from 26°C to 37°C in calcium-deficient medium results in a number of phenotypic alterations for Lcr^{+} Yersinia pestis known collectively as the low calcium response (42). The cells exhibit a cessation of growth (restriction) preceded by a doubling in cell number and a quadrupling of total cellular mass

resulting in elongated cells twice the size of normal cells (43). Electron micrographs of restricted cells show distinct abnormalties of cellular structures and apparent degeneration of integral structural components (43). Termination of stable ribonucleic acid (RNA) synthesis, but not necessarily messenger RNA synthesis, occurs within 2 hours of temperature shift (28). A subsequent reduction of adenylate energy charge and termination of deoxyribonucleic acid (DNA) synthesis occurs (97). The amount of DNA synthesis which occurs after temperature shift was calculated to be sufficient to carry out ongoing rounds of replication but not for the initiation of new rounds (97). The exact nature of restriction is not known, however it has been shown that the cessation of growth is an ordered metabolic event, does not involve guanosine tetraphosphate (magic spot I) or guanosine pentaphosphate (magic spot II), and probably represents a primary block in stable RNA synthesis (28).

Expression of virulence functions is closely associated with the low calcium response. Burrows and Bacon (23) showed that virulent strains of Y. pestis are able to synthesize two distinct antigens, V and W, both in vitro and in infected laboratory animals. These antigens were detected in crude cellular extracts of virulent, but not avirulent, Y. pestis by immunoprecipitation with serum from rabbits immunized by infection with virulent Y. pestis. The production of the virulence-associated antigens was subsequently shown to be a function of growth at 37°C in calcium-deficient media and was potentiated with high levels (at least 20 mM) of magnesium (16,58). Lawton et al (58) demonstrated that antibodies specific for V antigen passively protected mice from

intraperitoneal challenge with a lethal dose of Y. pestis, however no protection could be demonstrated using antibody to W antigen. The virulence-associated antigens have also been shown to be produced by Y. pseudotuberculosis (25) and Y. enterocolitica (26) during growth in calcium-deficient media at 37°C.

In addition to the production of V and W antigens, the enteropathogenic yersiniae also produce a specific set of outer membrane peptides (Yops) during the low calcium response (7,82). Yops are not produced at detectable levels at 26°C nor are they produced at 37°C in medium containing millimolar concentrations of calcium (8,35,81). At least 11 distinct Yops have been described by Straley and Bowmer and were given the nomenclature of YopA through YopK as described in Table 3 (81). YopA has been correlated with a multitude of effects including autoagglutination (78), mannose-resistant hemagglutination (54), surface-associated fibrillae expression (55,56), and adherence to human epithelial cells (44). It has also been demonstrated that unlike the other Yops, YopA is not influenced by calcium (35). Several other surface properties of the enteropathogenic yersiniae have been correlated with the low calcium response and presumably with expression of Yops. These properties include serum resistance (62,66,69) and increased hydrophobicity of the cell surface (62).

Using a solid medium made calcium-deficient by chelation with sodium oxalate and containing high levels of magnesium (which at 37°C permitted the growth of avirulent, but not virulent cells), Higuchi and Smith (47) showed that the spontaneous mutation rate of Y. pestis to

TABLE 3. Accepted nomenclature and approximate molecular weights of Yops expressed by the yersiniae.[a]

Accepted nomenclature [b]	Alternate nomenclature [c]	Approximate mol. weight [d]
YopA	Yop1	150-200 kdal
YopB	Yop2	44 kdal
YopC	Yop3	40 kdal
YopD	Yop4	34 kdal
YopE	Yop5	26 kdal
YopF	None	76 kdal
YopG	None	61.5 kdal
YopH	None	49.2 kdal
YopI	None	46.3 kdal
YopJ	None	31 kdal
YopK	None	21 kdal

a. All Yops reported to be produced by the yersiniae. Not all Yops are produced by all species or strains.

b. Nomeclature is taken from Straley and Bowmer (81).

c. Taken from Bölin *et al* (8).

d. As estimated by SDS-polyacrylamide gel electrophoresis.

avirulence was 10^{-4} mutations per bacterial generation. This fact coupled with the previous failure of researchers to detect the reversion of an avirulent strain back to virulent (22) prompted Ogg et al (65) to postulate the presence of "cytoplasmic factors" responsible for virulence. Early attempts to identify a plasmid responsible for virulence were not successful (60), and refinements in molecular biological techniques were necessary before a virulence-associated plasmid of ~42 megadaltons (~72 kb) was detected in Yersinia enterocolitica (40,99). This discovery was rapidly followed by the demonstration of similar plasmids in Y. pseudotuberculosis (41) and Y. pestis (6,34). These plasmids have been termed Lcr plasmids to denote the correlation of their presence with the low calcium response of the yersiniae (42).

The Lcr plasmids of all three Yersinia species are remarkably similar in size, reports ranging from 40-48 megadaltons are in the literature (3,6,34,40,41,72,99). Restriction analysis have also shown a great deal of homogeneity between various Lcr plasmids both in number and size of fragments using a number of different endonucleases (6,41,57,72,74). Similarities were strongest within different strains of a given species and between Y. pestis and Y. pseudotuberculosis. Sequence homologies between plasmids have been reported as 75-90% between strains of Y. enterocolitica (45,57). Only a 55% sequence homology was reported between the Lcr plasmids of Y. enterocolitica and Y. pestis (72), probably reflecting the divergence of these two species as evident by their relative lack of total DNA sequence homology (64).

The Lcr plasmid has received a great deal of attention by

researchers using recombinant approaches. An approximate 17 kilobase region of the plasmid has been shown by transposon mutagenesis (71) and Mu dl(Ap lac) fusions in Y. pestis (42) to be intimately involved in regulating the low calcium response. Insertions within this region conferred the Lcr$^-$ phenotype to cells. The mutations prevented induction of V antigen synthesis and rendered the cells avirulent in mice. Goguen et al reported that the regulatory region contains at least three transcriptional units which were induced at 37°C and were not affected by the Ca^{2+} concentration of the medium (42). Similar regulatory regions have been demonstrated on the Lcr plasmids of Yersinia enterocolitica (29,74) and of Yersinia pseudotuberculosis (74); mutations within these regions abolished the expression of Yops and V antigen. Insertion analysis of the Lcr plasmid has yielded several mutants defective in specific Yop expression (9,81), but insertions which specifically abolish V antigen expression have not been reported.

Although Yops are generally assumed to play a role in the virulence of enteropathogenic yersiniae, the role of Yops in Yersinia pestis infections has been a controversial subject. Yops are not expressed at detectable levels by Y. pestis when cultivated in vitro (73.82). It has been established that immune sera from convalescent plague patients contains antibody which recognizes the Yops of the enteropathogenic yersiniae (8,63) indicating that Y. pestis may express Yops in vivo. This phenomenon has also been demonstrated in laboratory animals which were infected with plague (8,63) and humans who were immunized with a commercially prepared plague vaccine (63). It is of interest to note that the latter immunization procedure uses formaldehyde-killed cells

prepared from cultures of Y. pestis. Since Y. pestis does not produce Yops in vitro, there should not be Yop antigens present in the vaccine to induce an anti-Yop immune response. The fact that a killed plague vaccine does induce an anti-Yop response makes the argument for the in vivo expression of Yops by Yersinia pestis somewhat less tenable.

In support of Yop expression by Y. pestis, it has been shown that transfer of the Lcr plasmid of Y. pestis to an Lcr^- strain of Y. pseudotuberculosis allows full expression of Yops, indicating that Lcr^+ Yersinia pestis possess the coding capacity for Yops (81,95). The fact that there are unique Yops produced by the transformants is compelling evidence that Yops are Lcr plasmid-encoded and that functional Yop genes are present in virulent strains of Y. pestis (81). The Lcr plasmid of Y. pestis has also been transformed into Escherichia coli (71,74) which was then able to express V antigen and Yops, albeit at greatly reduced levels relative to the enteropathogenic yersiniae. This phenomenon probably reflects unique chromosomal functions of the yersiniae which are necessary for the efficient transcription of Lcr genes as suggested by Cornelis et al (30).

CHAPTER I

(ARTICLE)

Modulation of the low calcium response via plasmid-plasmid interaction

by

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Modulation of the low-calcium response in *Yersinia pestis* via plasmid–plasmid interaction

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Virulent cells of *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* are known to exhibit a low-calcium response *in vitro* characterized by restriction of growth and induction of V antigen at 37°C in Ca^{2+}-deficient media (Lcr^+). A shared Lcr plasmid mediates these properties and encodes yersiniae outer membrane peptides (Yops) that are expressed in Lcr^+ *Y. pseudotuberculosis* and *Y. enterocolitica* but not *Y. pestis*. We present direct evidence here verifying that synthesis of major Yops in the former two species is, like V, repressed by Ca^{2+} and that these structures are located primarily in the outer membrane. We also verified that rabbits infected with live Lcr^+ *Y. pestis* can raise antibodies against V and Yops. Similar antisera, however, were recovered after immunization with sterile extracts of Ca^{2+}-starved Lcr^+ cells of *Y. pestis*. Results of immunoblots obtained with these antisera showed that restricted *Y. pestis* produced Yops of about 46 kDa (YopB) and 44 kDa (YopC) after shiftup by addition of Ca^{2+} at 37°C or reduction of temperature to 26°C. It is established that virulent cells of *Y. pestis* also possess a unique plasmid known to mediate pesticinogeny (Pst^+). Restricted Lcr^+, Pst^- *Y. pestis* expressed YopB and YopC plus additional 76 kDa (YopF), 48 kDa (YopH), 36 kDa (YopD), 32.5 kDa (YopJ), and 27 kDa (YopE) outer membrane structures at concentrations comparable to those in Ca^{2+}-starved *Y. pseudotuberculosis* and *Y. enterocolitica*. These findings indicate that carriage of the Pst plasmid prevents expression of the Lcr plasmid-mediated Yops in wild type *Y. pestis*.

Key words: Plasmids; low-calcium response; yersiniae outer membrane peptides (Yops); V antigen; *Yersinia*.

Introduction

Yersinia pestis, the causative agent of bubonic plague, and closely related *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* exhibit a unique low-calcium response characterized by restriction of cell division at 37°C in Ca^{2+}-deficient media accompanied by maximum induction of V antigen (Lcr^+). These properties are mediated by a shared approximate 70 kb Lcr plasmid that also encodes a series of yersiniae outer membrane peptides[1,2] (Yops). The latter exist as major constituents of Ca^{2+}-starved Lcr^+ *Y. pseudotuberculosis*[3] and *Y. enterocolitica*[4] but are not detectable in comparable preparations of *Y. pestis*.[3] Nevertheless, sera obtained from man or animals following convalescence from plague often contain antibodies directed against Yops[5,6] prompting the suggestion that these structures undergo induction in *Y. pestis* during growth *in*

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vivo.[2] Transfer of the Lcr plasmid of *Y. pestis* to Lcr⁻ *Y. pseudotuberculosis* permitted expression of a full complement of Yops which are conveniently distinguished by molecular weight.[7] Elimination of the Lcr plasmid[1,2] or occurrence of certain mutations within either its 17 kb Yop[2,5,7-10] or adjacent V[11] regulatory regions result in respective loss or superrepression of these factors, acquisition of Ca^{2+}-independence, and avirulence. Similarly, mutation in some but not all structural genes for Yops[7,12,13] and probably that for V[11] causes significant reduction or outright loss of virulence.

A second virulence factor unique to *Y. pestis* is the ability to produce the bacteriocin pesticin[14,15] and genetically-linked[16,17] coagulase and fibrinolytic activities[18] (Pst^+). These properties are mediated by an approximate 10 kb Pst plasmid not present in *Y. pseudotuberculosis* or *Y. enterocolitica*.[19,20] Loss of this plasmid in *Y. pestis* results in significantly reduced virulence in mice infected by peripheral routes of injection[21] and disappearance of two major outer membrane peptides possibly representing coagulase and fibrinolysin.[22] The major purpose of this report is to present evidence demonstrating that mutation of Lcr^+ cells of *Y. pestis* to Pst^- permits typical full expression of Yops as occurs in Lcr^+ *Y. pseudotuberculosis* and *Y. enterocolitica*. In addition, conditions permitting limited synthesis of Yops in Lcr^+, Pst^+ *Y. pestis* are defined and direct evidence is presented showing that most of these structures, like V, are repressed by Ca^{2+}

Results

As reported by others, both anti-V[23] and anti-Yops[5,6] were present in antisera raised against live Lcr^+ cells of *Y. pseudotuberculosis* (Fig. 1(A)) and *Y. pestis* (Fig. 1(B)). However, comparable immunoblots prepared with antisera obtained after immunization with sterile extracts of Ca^{2+}-starved Lcr^+ cells of *Y. pestis* also contained anti-V and anti-Yops (Fig. 1(C)). This result indicates that Yops or antigenically related components were present in the sterile extracts even though only one such structure of about 46 kDa (YopB) was detected in whole restricted cells of *Y. pestis*. Nevertheless, the antisera generated against live Lcr^+ yersiniae often contained a broader spectrum of antibodies directed against minor components (lane 1 of Fig. 1(A) and 1(B)) than did those raised with sterile cell-free extracts (lane 1 of Fig. 1(C)). These antisera did not exhibit significant reactivity against Lcr^- yersiniae and were used in subsequent experiments concerned with expression of Yops by *Y. pestis*.

Lcr^+ yersiniae were cultivated under restrictive conditons and used for production of purified samples of cytoplasm, inner membranes, and outer membranes. These preparations were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained directly. At least five Yops were present in outer membranes of Lcr^+ but not Lcr^- *Y. pseudotuberculosis* (Fig. 2(A)); concentrations of these structures in inner membranes and cytoplasm were significantly reduced. Identical results occurred with the four detectable Yops of Lcr^+ *Y. enterocolitica* (Fig. 2(B)) and, as expected, Yops were not observed in comparable preparations of *Y. pestis* (Fig. 2(C)). These findings verify that Yops are essentially restricted to the outer membrane of Lcr^+ *Y. pseudotuberculosis* and *Y. enterocolitica* as opposed to V which is known to be present at significant levels only within cytoplasm[3,22] and culture supernatant fluids.[23,24] Similar determinations were performed to directly demonstrate the ability of Ca^{2+} to repress production of Yops. Growth at 26°C without the cation or at 37°C in its presence did not significantly repress appearance of the approximate 200 kDa YopA which is known to be regulated independently[12] of V and the remaining Yops. However, these environments prevented expression of the remaining detectable Yops of *Y. enterocolitica* (Fig. 3(A)) and

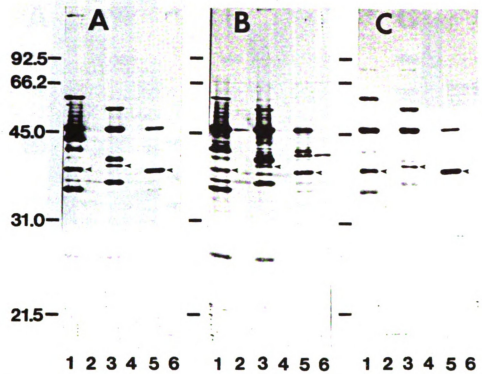

Fig. 1. Expression by immunoblotting of Yops and V (arrowheads) in whole cells of (lane 1) Lcr$^+$ *Y. pseudotuberculosis* PB1, (lane 2) Lcr$^-$ *Y. pseudotuberculosis* PB1, (lane 3) Lcr$^+$ *Y. enterocolitica* WA, (lane 4) Lcr$^-$ *Y. enterocolitica* WA, (lane 5) Lcr$^+$, Pst$^+$ *Y. pestis* KIM, and (lane 6) Lcr$^-$, Pst$^+$ *Y. pestis* KIM. Antisera were raised against live cells of Lcr$^+$ *Y. pseudotuberculosis* PB1 (A) and Lcr$^+$, Pst$^+$ *Y. pestis* KIM (B) or against a sterile extract of Ca^{2+}-starved cells of Lcr$^+$, Pst$^+$ *Y. pestis* KIM (C) as described in the text. Color development was minimized to favor selective expression of V. Molecular weight indicators are in kilodaltons.

Y. pseudotuberculosis (Fig. 3(B)). These results verify that the detectable lower molecular weight Yops are regulated via the low-calcium response and provide a basis for directly demonstrating the existence of these structures in Lcr$^+$ *Y. pestis*.

Although YopB was often detected in Lcr$^+$ *Y. pestis* by immunoblotting (Fig. 1), appearance of this structure generally required metabolic shiftup such as storage of cultures overnight in the cold. To further define the effect of Ca^{2+} and temperature on Lcr plasmid function in *Y. pestis*, Lcr$^+$ organisms were restricted in parallel cultures for 6 h at which time growth and significant net macromolecular synthesis had ceased.[25,26] A culture was then shifted to 26°C and two others were retained at 37°C either without or with added Ca^{2+} (4.0 mM). As shown in immunoblots, only V was detectable in organisms maintained at 37°C less Ca^{2+} whereas shiftup by addition of the cation resulted in expression of YopB and 44 kd YopC (Fig. 4). Similarly, shiftup by transfer to 26°C promoted appearance of YopC with later production of YopB after 4 h of growth. Prior restriction was necessary to permit detection of these Yops in *Y. pestis* as shown by their absence in cultures maintained at 26°C or at 37°C with 4.0 mM Ca^{2+} (not illustrated). These results also demonstrated that V did not undergo detectable destruction during vegetative growth following shiftup.

The possibility remained, however, that Yops in Lcr$^+$ *Y. pestis* do undergo turnover during restriction but become stabilized upon shift to conditions permitting subsequent

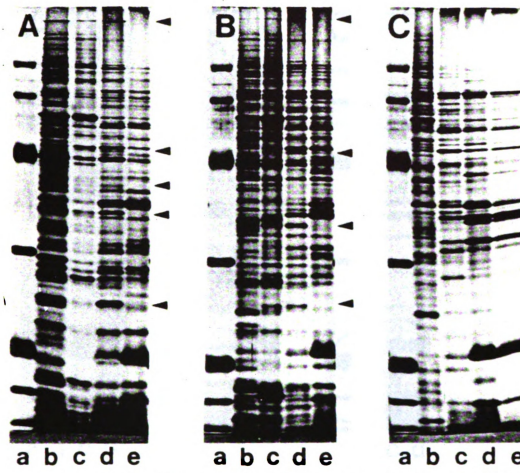

Fig. 2. Silver-stained preparations of (A) *Y. enterocolitica* WA, (B) *Y. pseudotuberculosis* PB1, and (C) *Y. pestis* KIM; gels illustrate (lane a) molecular weight markers from top to bottom of 92.5, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa, respectively, (lane b) Lcr^+ cytoplasm, (lane c) Lcr^+ inner membranes, (lane d) Lcr^+ outer membranes, and (lane e) Lcr^- outer membranes. All yersiniae were cultivated at 37°C in chemically defined medium containing 20 mM Mg^{2+} and no added Ca^{2+}.

multiplication. The only known outer membrane-specific proteolytic functions unique to *Y. pestis* are the coagulase and fibrinolytic activities mediated by the Pst plasmid. To verify that carriage of this plasmid prevents expression of Yops in Lcr^+ *Y. pestis*, we first showed that these peptides were not detectable in immunoblots of three Lcr^+, Pst^+ isolates but occurred in three Lcr^+, Pst^- strains at concentrations evidently equivalent to those in Lcr^+ *Y. pseudotuberculosis* and *Y. enterocolitica* (Fig. 5(A)). This phenomenon was also shown with isogenic mutants of *Y. pestis* EV76 (Fig. 5(B)) where only V was observed in the Lcr^+, Pst^+ parent whereas this antigen plus major Yops of 46 kDa (YopB), 44 kDa (YopC), and 36 kDa (YopD) and probably those of higher molecular weight were present in the Lcr^+, Pst^- isolate. This determination was repeated with the antiserum raised against sterile cell-free extract of Lcr^+, Pst^+ *Y. pestis* in order to directly compare the number and molecular weights of Yops produced by the three yersiniae and to further illustrate the role of Ca^{2+} as repressor. The antiserum permitted detection of at least six Yops in Lcr^+ cells of *Y. pseudotuberculosis* (Fig. 6(A)), *Y. enterocolitica* (Fig. 6(B)), and Pst^- *Y. pestis* (Fig. 6(D)); expression of these structures was markedly inhibited by Ca^{2+} or carriage of

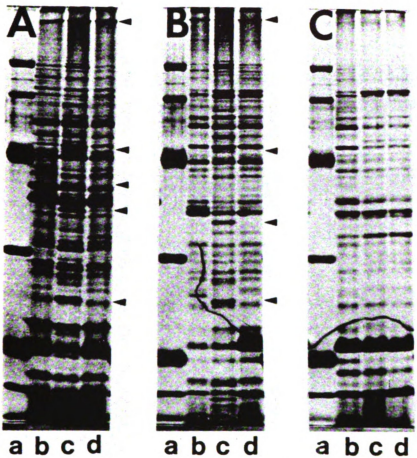

Fig. 3. Silver-stained outer membrane preparations of Lcr$^+$. (A) *Y. enterocolitica* WA, (B) *Y. pseudotuberculosis* PB1, (C) *Y. pestis* KIM; gels illustrate (lane a) molecular weight markers as in Fig. 2, (lane b) sample after growth at 37°C with 4.0 mM Ca^{2+}, (lane c) sample after growth at 37°C without added Ca^{2+}, and (lane d) sample after growth at 26°C without added Ca^{2+}.

the Pst plasmid (Fig. 6(C)). The additional Yops observed in Lcr$^+$, Pst$^-$ *Y. pestis* by this process were about 76 kDa (YopF) and 26 kDa (YopE). All of these structures plus another Yop of 48 kDa (YopH) were directly detected in outer membranes of Ca^{2+}-starved Lcr$^+$, Pst$^-$ but not Lcr$^+$, Pst$^+$ cells of *Y. pestis* (Fig. 7). Accordingly, loss of the Pst plasmid of wild type *Y. pestis* permits expression of at least YopB, YopC, YopD, YopE, YopF, YopH, and YopJ, all of which can be repressed by Ca^{2+} as are their analogues in Lcr$^+$ *Y. pseudotuberculosis* and *Y. enterocolitica*.

Discussion and conclusions

The two methods used in this study to identify Yops are not interchangeable and caution is required when comparing findings obtained by these procedures. Assay by immunoblotting is generally more sensitive than that performed by direct staining and the former can be accomplished with complex samples including solubilized whole cells. Nevertheless, this method is only qualitative and, in our experience, not dependable with Yops of extreme high and low molecular weights. Direct staining does, of course, provide semi-quantitative information but requires appropriate frac-

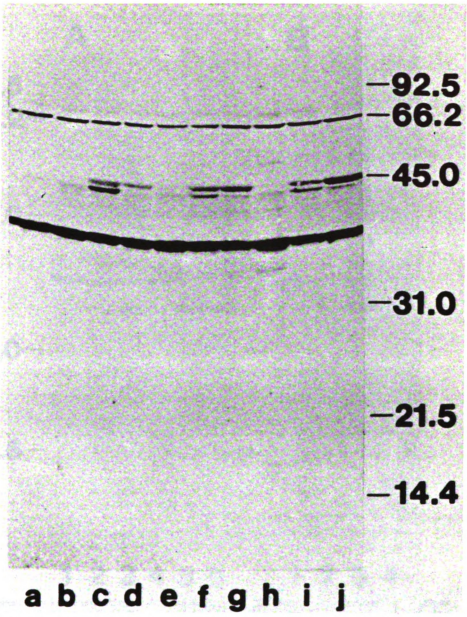

Fig. 4. Expression by immunoblotting of YopB (46 kDa), YopC (44 kDa), and V (37 kDa) by Lcr$^+$, Pst^r cells of *Y. pestis* KIM (lane a) after restriction at 37°C in Ca^{2+}-deficient medium for 4 h and then (lane b) 1 h without change, (lane c) 1 h after addition of 4.0 mM Ca^{2+} at 37°C, (lane d) 1 h after shift to 26°C without addition of Ca^{2+}, (lane e) 2 h without change, (lane f) 2 h after similar addition of Ca^{2+}, (lane g) 2 h after similar temperature shift, (lane h) 4 h without change, (lane i) 4 h after similar addition of Ca^{2+}, and (lane j) 4 h after similar temperature shift; values for molecular weight markers are in kilodaltons. Antiserum was prepared against live Lcr$^+$ cells of *Y. pseudotuberculosis* PB1.

tionation of whole cells prior to electrophoresis in order to remove bulk protein thereby permitting acceptable visualization of salient individual peptides. This process as performed by osmotic lysis and isopycnic centrifugation did permit localization of Yops to the outer membrane although the possibility remains that at least some of these structures are in equilibrium with or are deposited within the outer membrane but fulfill physiological roles in cytoplasm or the extracellular environment. This

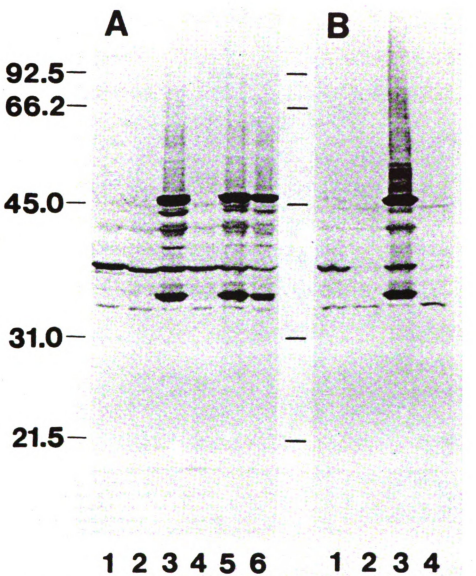

Fig. 5. Expression by immunoblotting of assorted Yops and V (37 kDa) in (A) unrelated Lcr$^+$ *Y. pestis* strains (lane 1) KIM (Pst$^+$), (lane 2) EV76 (Pst$^+$), (lane 3) G25 (Pst$^-$), (lane 4) M23 (Pst$^+$), (lane 5) 019 (Pst$^-$), and (lane 6) 2C (Pst$^-$) and (B) isogenic (lane 1) Lcr$^+$, Pst$^+$; (lane 2) Lcr$^-$, Pst$^+$; (lane 3) Lcr$^+$, Pst$^-$; and (lane 4) Lcr$^-$, Pst$^-$ isolates of *Y. pestis* EV76. Antiserum was prepared against live Lcr$^+$, Pst$^+$ cells of *Y. pestis* KIM and color development was prolonged to emphasize expression of Yops.

situation is distinct from that of V which exists primarily in cytoplasm[3,22] or in culture supernatant fluid[23,24] and has only a transient association with the outer membrane as judged by our ability to detect its presence therein by immunoblotting (unpublished observations) but not by direct staining.[3,22]

A third acutely perceptive method for detecting the presence of Yops is by immunization. It is generally established that ability to produce antibodies against a given peptide can constitute a far more sensitive assay system for that structure than would direct staining or immunoblotting. Accordingly, the existence of anti-Yops in convalescent plague sera was correctly interpreted as evidence demonstrating that

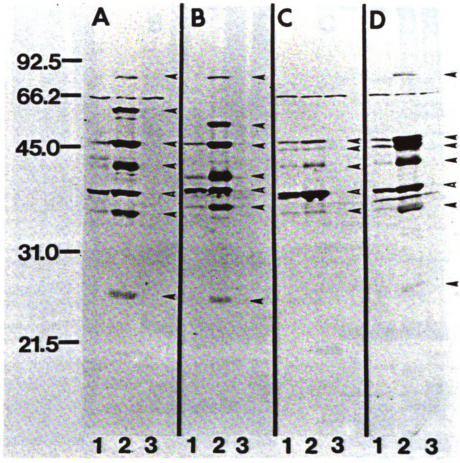

Fig. 6. Expression by immunoblotting of assorted Yops (closed arrowheads) and V (open arrowheads) in (A) *Y. pseudotuberculosis* PB1, (B) *Y. enterocolitica* WA, (C) Pst$^+$ *Y. pestis* KIM, and (D) Pst$^-$ *Y. pestis* KIM all grown at 37°C with 20 mM Mg^{2+}; (lane 1) Lcr$^+$ yersiniae with 4.0 mM Ca^{2+}, (lane 2) Lcr$^-$ yersiniae without added Ca^{2+}, and (lane 3) Lcr$^-$ yersiniae without added Ca^{2+}. Molecular weight markers are in kilodaltons and antiserum was raised against a sterile extract of Ca^{2+}-starved cells of Lcr$^+$, Pst$^+$ *Y. pestis* KIM. Color development was extended to favor expression of Yops.

Lcr$^+$ *Y. pestis* produces these structures *in vivo*[5,6] However, our finding that anti-Yops were similarly raised by immunization with sterile extracts of restricted *Y. pestis* indicates that at least equivalent levels of these peptides are produced *in vitro*. Indeed, this discovery precludes the suggestion that Yops are only expressed by *Y. pestis* in the mammalian host[2] although the possibility remains that some *in vivo* environment promotes full induction comparable to that observed *in vitro* for Lcr$^+$ *Y. pseudotuberculosis* and *Y. enterocolitica*.

Ability of the immune system to respond to trace amounts of foreign protein acquires real significance in view of the explanation provided in this report for the cryptic nature of Yops in *Y. pestis*. Reduced levels of a few such structures were detected in immunoblots of Lcr$^+$, Pst$^+$ isolates, especially after metabolic shiftup, and these components plus any others present below detectable levels may have prompted synthesis of corresponding antibodies. It is not yet clear why carriage of the Pst plasmid prevents net synthesis of Yops. We favor the hypothesis that these peptides undergo hydrolysis mediated by the plague coagulase and fibrinolytic activities.

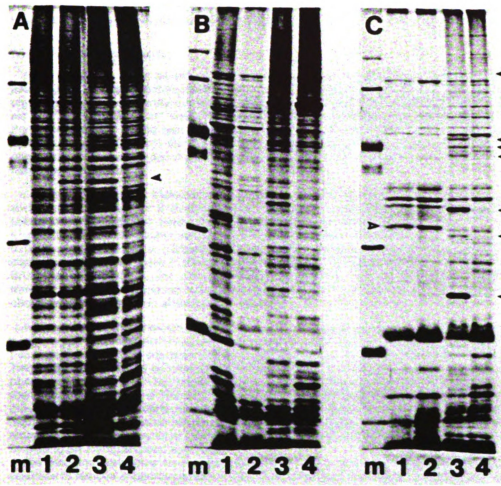

Fig. 7. Silver-stained preparations of (A) cytoplasm, (B) inner membranes, and (C) outer membranes of (lane 1) Lcr$^-$, Pst$^+$; (lane 2) Lcr$^+$, Pst$^+$; (lane 3) Lcr$^+$, Pst$^-$; and (lane 4) Lcr$^-$, Pst$^-$ cells of *Y. pestis* KIM; molecular weight markers (lane m) are as in Fig. 2. Solid arrowheads refer to Yops in outer membranes and V in cytopolasm; open arrowhead represents the smaller of the two known Pst$^+$-specific outer membrane peptides.

However, proof of this occurrence will require demonstration that the structures are indeed synthesized and then degraded and that they remain intact in Pst plasmid-mutants lacking only this function. In the absence of this information, the possibilities remain that the block in Yop expression in Pst$^+$ yersiniae occurs at the level of transcription or translation. In any event, the failure of Lcr$^+$, Pst$^+$ organisms to fully express Yops is not artifactual due to occurrence of degradation during separation and purification of outer membranes because whole bacteria also lacked detectable Yops as shown by immunoblotting. Accordingly, such degradation would have to occur concomitantly with synthesis, a situation that might lead to generation of possibly antigenic fragments still capable of initiating a specific immune response.

Although a number of biological roles have been proposed for Yops and V, the significance of the low-calcium response in promoting disease has not yet been resolved.[1,2] It is now evident that this environment promotes release of soluble Yops by Lcr$^+$ *Y. pseudotuberculosis* and *Y. enterocolitica*[27] thereby increasing the number of their possible modes of action. Further comparative study of Lcr$^+$, Pst$^+$ and Lcr$^+$,

Pst$^-$ cells of *Y. pestis* may reveal the function of these peptides and demonstrate why their full expression is evidently not required for Pst$^+$ yersiniae to cause bubonic plague.

Materials and methods

Bacteria. Unless stated otherwise, the type strains used throughout this study were Lcr$^+$ *Y. pestis* KIM, *Y. pseudotuberculosis* PB1/+, and *Y. enterocolitica* WA. The former is non-pigmented[28] and thus avirulent in normal mice except by intravenous injection;[29] the remainder are wild type. Isogenic Lcr$^-$ yersiniae were selected at 37°C on magnesium oxalate agar[30] and Pgm$^-$ mutants of *Y. pestis* were obtained by use of hemin agar.[28] Pst$^-$ mutants were enriched by subculture at 5°C where the Pst-plasmid is lost from the bacterial population (unpublished observations). Properties and origins of these yersiniae[3,7,9–11,19,22] and other strains of *Y. pestis*[2,19] have been described.

Media and cultivation. Yersiniae were incubated at 26°C for 1 day (*Y. pseudotuberculosis* and *Y. enterocolitica*) or 2 days (*Y. pestis*) on slopes of Tryptose blood agar base (Difco Laboratories, Detroit, MI), removed in 0.033 M potassium phosphate buffer, pH 7.0, and used to inoculate liquid chemically defined medium[26] or fermenter medium[24] at an optical density at 620 nm of 0.1. The former, containing 20 mM Mg^{2+} and either 4.0 mM or no added Ca^{2+}, was aerated in Erlenmeyer flasks (10% vol/vol) on a model G76 gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 200 rpm for at least a single culture transfer before use in preparing subcultures for growth at 37°C. The latter medium was used to obtain organisms for use in absorption of antisera.

Antisera. Antibodies directed against Yops and V were raised by either injection of rabbits with live Lcr$^+$ yersiniae according to an established protocol[23] or by injection of a crude cell-free extract mixed with complete Freund's adjuvant as described for preparation of anti-V.[31] The extract was prepared by washing restricted Lcr$^+$, Pst$^+$ yersiniae twice in 0.033 M potassium phosphate buffer, pH 7.0 by centrifugation at 4°C (11 000 *g* for 30 min); the organisms were then suspended in 0.05 M Tris · HCl buffer, pH 7.8 and disrupted at 20 000 lb/in^2 in a French pressure cell. Cellular debris was removed by centrifugation (11 000 *g* for 30 min) and the resulting extract was either saturated with $CHCl_3$ or passed through a 0.22-μm pore size membrane filter (Millipore Corp., Bedford, MA) to eliminate viable bacteria. Sterility was verified by plating on Tryptose blood agar base.

The resulting sera were then exhaustively absorbed with disrupted and lyophilized Lcr$^-$ yersiniae to remove antibodies directed against common determinants.[31] These antisera reacted in immunoblots with both Yops and V which was located on the basis of a molecular weight of about 37 kDa.[3,11,22,24]

Assay of Yops. Preparations of outer membranes, inner membranes, and cytoplasm were isolated by osmotic lysis and isopynic centrifugation as previously described where contamination of outer membrane with inner was about 3% and the reverse ranged between 5% to 38%.[3] Immunoblotting was performed as already defined[24] where whole cells were rapidly solubilized by addition to SDS-PAGE buffer thereby causing immediate denaturation of putative hydrolytic enzymes capable of degrading Yops. Methods used for SDS-PAGE have been presented.[3,24] Protein in solution was determined by the procedure of Lowry *et al.*[32] and located in gels by silver staining.[33] A standardized system of nomenclature based on use of the same Lcr plasmid as expressed in *Y. pseudotuberculosis*[7] was used for definition of Yops. These values differed from those obtained in this work by no more than about 2000 daltons.

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CHAPTER II

(ARTICLE)

Post-translational regulation of Lcr plasmid-mediated peptides in pesticinogenic *Yersinia pestis*

by

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ABSTRACT

The low calcium response of wild type Yersinia pestis, the causative agent of bubonic plague, and of enteropathogenic Yersinia pseudotuberculosis and Yersinia enterocolitica is known to be mediated by a shared Lcr plasmid of about 70 kb. At 37°C in Ca^{2+}-deficient medium, this element promotes restriction of growth with concomitant production of virulence functions including the common V antigen and a set of yersiniae outer membrane peptides termed Yops (Lcr^+). The latter are expressed by the enteropathogenic species but not by wild type Y. pestis which possesses a unique 10 kb Pst plasmid associated with pesticinogeny (Pst^+). We show in this report that, after pulse with ^{35}S-methionine, peptides with molecular weights corresponding to Yops of 78, 47, 45, 44, 36 and 26 kDa are synthesized during the low calcium response by both Lcr^+,Pst^+ and Lcr^+,Pst^- cells of Y. pestis. Although stable in the latter, radioactivity in Yops of wild type was rapidly chased into lower molecular weight degradation products. At least four soluble peptides, including V, were also labeled during starvation for Ca^{2+}; these structures were stable in both Lcr^+,Pst^+ and Lcr^+,Pst^- yersiniae. These findings suggest that a product encoded by the pesticin plasmid of Y. pestis is required for post-translational regulation of outer membrane but not soluble peptides mediated by a second unrelated Lcr plasmid.

INTRODUCTION

Wild type cells of Yersinia pestis harbor a cryptic ~100 kb plasmid, a ~70 kb Lcr plasmid that mediates the low calcium response (Lcr^+), and a ~10 kb Pst plasmid that promotes pesticinogeny (Pst^+). Enteropathogenic Yersinia pseudotuberculosis and Yersinia enterocolitica share the Lcr plasmid but lack the cryptic plasmid and the Pst plasmid which is required for the invasive function that accounts for the severe symptoms of bubonic plague (1,2,3). The common Lcr plasmid encodes determinants that are expressed at 37°C in the absence of Ca^{2+} where they cause a metabolic stepdown characterized in Y. pestis by reduction of adenylate energy charge, shutoff of stable RNA synthesis, and cessation of cell division (4,5). This form of restriction, however, is requisite for maximum induction in vitro of putative virulence factors (6,7,8,9) also encoded on the Lcr plasmid (10,11,12). These structures include the plague virulence or V and W antigens (13,14,15) and a set of yersiniae outer membrane peptides (7,10,16,17,18,19). Known products encoded by the Y. pestis-specific Pst plasmid are the bacteriocin pesticin (20,21,22) and particulate coagulase plus fibrinolytic activities (23) probably both mediated by a single plasmin activator gene (Jon Goguen, personal communication).

Although the Lcr plasmid of Y. pestis and those of the enteropathogenic yersiniae exhibit remarkably similar homology (18,19), the low calcium response of these species is distinct. For example, when restrictied at 37°C under comparable conditions with 20 mM Mg^{2+}, multiplication of Y. pestis ceases almost immediately whereas cells of

the enteropathogenic yersiniae undergo residual divisions or continue susstained growth at increased doubling times (1,24). Furthermore, all species produce V antigen in this environment whereas only the enteropathogenic species express significant levels of Yops (16). Whereas these differences in kinetics of growth following shift to restrictive conditions reflect the source of the Lcr plasmid (10), inability of Y. pestis to fully express Yops is correlated with carriage of the Pst plasmid (25).

It is established that the Lcr plasmid of Y. pestis encodes the ability to produce Yops (10,17) and that, after growth in vitro, these structures exist in this species at reduced levels as antigens (25) possibly in the form of degradation products (26). It is not known if the block in net synthesis of Yops by Lcr^+,Pst^+ Y. pestis occurs at the level of transcription, translation, or elsewhere. The purpose of this report is to present evidence demonstrating that Yops are synthesized with equal facility by both Lcr^+,Pst^+ and Lcr^+,Pst^- yersiniae but that these structures undergo immediate post-translational degradation in the former. This modification did not occur with V or three other soluble peptices produced during expression of the low calcium response.

RESULTS

Radiolabeling of Lcr^+,Pst^+ yersiniae

After Ca^{2+}-starved Lcr^+,Pst^+ cells of Y. pestis were pulsed for 1 minute with ^{35}-methionine, a discrete set of peptides contained radioactivity whereas bulk cellular protein remained unlabeled (Fig. 1A). These labeled structures were assumed to represent primary gene products since they were synthesized during brief exposure to ^{35}S-methionine and the extent of incorporated radioactivity did not incrase in intenstiy after chase with unlabeled methionine.

Seven labeled peptides (p78, p47, p45, p44, p36, p32, and p26) with molecular weights similar to established Yops of Y. pestis (Table 1) underwent rapid degradation as judged by disappearance of radioactivity upon chase with unlabeled methionine. In most cases, previously incorporated radioisotope became chased after 15 to 30 minutes into low molecular weight structures that accumulated at the dye front of the gel. However, p36 was still present at decreased but significant levels after chase for 30 minutes and p26 disappeared almost immediately. Another labeled peptide (p24) not correlated with an established Yop also underwent immediate degradation. In contrast to the above, four radioactive peptides (p70, p56, p38, and p20) remained stable within intact cells for at least 1 hour. Of these, p38 was equated with V antigen (Table 1) on the basis of molecular weight.

Labeling of Lcr^+,Pst^+ yersiniae under conditions that allowed constant incorporation of ^{35}S-methionine (Fig. 1B) promoted continuous increase of radioactivity in the four stable peptides. In contrast, the

FIGURE 1. Autoradiogram of trichloroacetic acid-precipitated material from *Yersinia pestis* KIM (Lcr^+,Pst^+) labeled with ^{35}S-methionine during restriction of growth at 37°C in calcium-deficient medium. Peptides were separated by 12.5% SDS-PAGE. Incorporated label chased with 0.1 mM unlabeled methionine for the indicated times (A); 0 min (lane 1), 1 min (lane 2), 2 min (lane 3), 4 min (lane 4), 8 min (lane 5), 15 min (lane 6), 30 min (lane 7), and 60 min (lane 8). Constant incorporation of radioactivity with sufficient carrier (2.0μM) to permit labeling for 30 min (B); 0.5 min (lane 1), 1 min (lane 2), 2 min (lane 3), 4 min (lane 4), 8 min (lane 5), 15 min (lane 6), and 30 min (lane 7). Open arrowheads mark peptides that are stable for at least 1 h and closed arrowheads mark peptides, presumably Yops, that undergo degradation within 1 h; the peptide marked with an arrow is a transient degradation product discussed in the text.

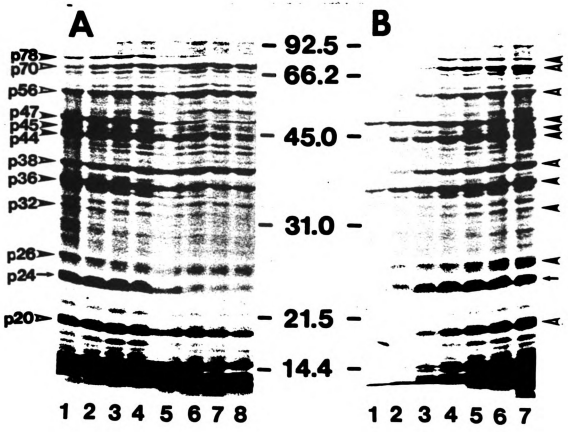

FIGURE 1. Autoradiogram of trichloroacetic acid-precipitated material from Yersinia pestis KIM (Lcr^+,Pst^+) labeled with ^{35}S-methionine during restriction of growth at 37°C in calcium-deficient medium.

TABLE 1. Properties of peptides produced by *Yersinia pestis* KIM during cultivation at 37°C in Ca^{2+}-deficient medium.

Designation	Stability in Lcr^+, Pst^+ cells	Location in Lcr^+, Pst^- cells	Alternate nomenclature[10]	Reported molecular weight[10]
p78	Instable	OM [a]	YopF	76,000
p70	Stable	OM,C	None	None
p56	Stable	C	None	None
p47	Instable	OM	YopH	49,200
p45	Instable	OM	YopB	44,000
p44	Instable	OM	YopC	42,000
p38	Stable	C	V antigen	38,000
p36	Instable	OM	YopD	34,400
p32	Instable	OM	YopJ	31,000
p26	Instable	OM	YopE	25,000
p24	Instable	Not produced	None	None
p20	Stable	C	None	None

a. OM indicates outer membrane fraction; C indicates cytoplasmic fraction.

concentrations of the eight unstable peptides approached steady-state equilibrium. Again, radioactive low molecular weight material accumulated throughout the course of labeling.

Radiolabeling of Lcr^+,Pst^- yersiniae

The determination described above was performed with an isogenic strain of *Y. pestis* cured of the Pst plasmid. In this case, eleven radiolabeled peptides remained stable for at least 1 hour in intact organisms (Fig. 2A). The labeled peptide p24 was not detected nor did significant radioactive low molecular weight material migrate at the gel front. During constant incorporatin of label (Fig. 2B), the concentration of all 11 peptides increased continuously without significant appearance of radioactivity in low molecular weight material.

Subcellular fractionation of labeled yersiniae

Cells of Lcr^+,Pst^+ and Lcr^+,Pst^- yersiniae were cultivated at 37°C without Ca^{2+}, pulsed with ^{35}S-methionine, and then chased for 1 hour with unlabeled methionine. This process resulted in recovery of yersiniae containing significant incorporated radioactivity only in stable peptides or in distinct products of degradation. These bacteria were fractionated into samples of outer membrane and cytoplasm and then prepared for autoradiography by electrophoresis in parallel with samples of culture supernatant fluids and trichloroacetic acid-precipitated whole cultures.

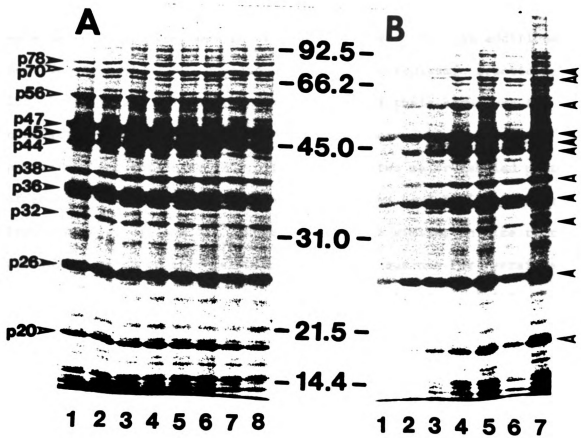

FIGURE 2. Autoradiogram of trichloroacetic acid-precipitated material from Yersinia pestis KIM (Lcr^+,Pst^-) labeled with ^{35}S-methionine during restriction of growth at 37°C in calcium deficient medium. Legend is as described for Fig. 1.

Results indicated that stable p70, p56, p38, and p20 occurred primarily in cytoplasm of both Lcr^+,Pst^+ and Lcr^+,Pst^- organisms (Fig. 3; lanes 3,4) although low levels of p38 were present in supernatant fluids (Fig. 3; lanes 7,8) and significant levels of p70 were detected in outer membranes (Fig. 3; lanes 5,6). An additional peptide of about 31 kDa appeared only in the cytoplasmic fraction of the Lcr^+,Pst^+ isolate (Fig.3; lane 3); the size of theis structure is consistant with that of the major V antigen degradation product of pesticinogenic yersiniae (25). In addition, two major labeled peptides of about 33 and 36 kDa were present only in the Lcr^+,Pst^+ outer membrane fraction (Fig. 3; lane 5). These peptides are similar in size to those previously reported in outer membrane of Pst^+ but not Pst^- yersiniae (25,27) and may reflect particulate plasmin activator activities.

All seven peptides that were stable in Lcr^+,Pst^- but not Lcr^+,Pst^+ yersiniae were localized within the outer membrane of the former (Fig. 3; lanes 5,6). Furthermore, large amounts of p47 and p36 were also present within the corresponding culture supernatant fluid along with smaller amounts of the remaining five outer membrane structures (Fig. 3; lane 8). A trace level of p36 was also detedted in the cytoplasmic fraction of Lcr^+,Pst^- bacteria (Fig. 3; lane 4); none of the other outer membrane proteins were visible therein.

Degradation products of Lcr^+,Pst^+ yersiniae

The radioactive low molecular weight products that accumulated during chase in Lcr^+,Pst^+ yersiniae were present exclusively in the culture supernatant fluid (Fig 3, lane 7). The size of these peptides

FIGURE 3. Autoradiogram of subcellular fractions of Lcr^+,Pst^+ and Lcr^+,Pst^- cells of Yersinia pestis strain KIM. Trichloroacetic acid-precipitated whole cultures (lanes 1,2), cytoplasmic fractions (lanes 3,4), outer membranes (lanes 5,6), and culture supernatant fluids (lanes 7,8). Lcr^+,Pst^+ preparations are shown in lanes 1, 3, 5, and 7 and Lcr^+,Pst^- preparations are given in lanes 2, 4, 6, and 8. Peptides were separated on 12.5% SDS-PAGE. Stable peptides found only in Lcr^+,Pst^- cells are marked with closed arrowheads and stable peptides present within cytoplasm of both strains are shown with open arrowheads; and Lcr^+,Pst^+-specific degradation product of V antigen is marked by an arrow.

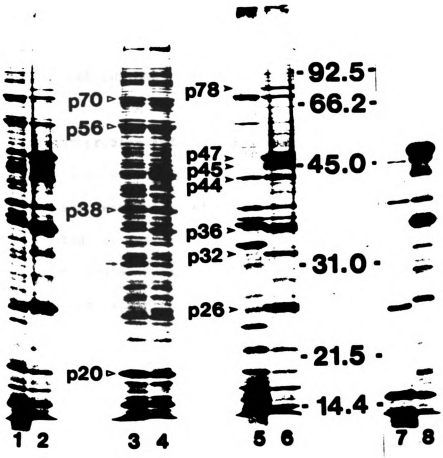

FIGURE 3. Autoradiogram of subcellular fractions of Lcr^+,Pst^+ and Lcr^+,Pst^- cells of Yersinia pestis strain KIM.

was 15 kDa or less (Fig. 4). They were absent in comparable preparations of $Lcr^+.Pst^-$ organisms which contained the larger soluble precursor structures described above.

DISCUSSIONS AND CONCLUSIONS

An important problem and one that is especially challenging to those concerned with virulence in yersiniae is defining the significance of the low calcium response. Before significant progress in this area can be anticipated, it may become necessary to distinguish between Lcr plasmid-mediated virulence factors per se and components involved in the performance and regulation of restriction. Direct staining of gels has yielded important information in comparing profiles of peptides expressed by Lcr^+ organisms cultivated with and without Ca^{2+} (25). This approach permits detection of unique peptides undergoing pronounced induction but those produced at low levels are often difficult to distinguish among bulk cellular protein. Another limitation of direct staining is the inability of this process to identify salient peptides synthesized during both the low calcium response and normal vegetative growth. A second approach for defining the low calcium response is by use of antiserum from previously infected or immunized mammals (17,25,29). Specific antisera are useful for generating immunoprecipitates (15) or for immunoblotting where reactive peptides can be detected in picogram quantities (30). However, antibodies present in sera raised against whole or fractionated Lcr^+ yersiniae can

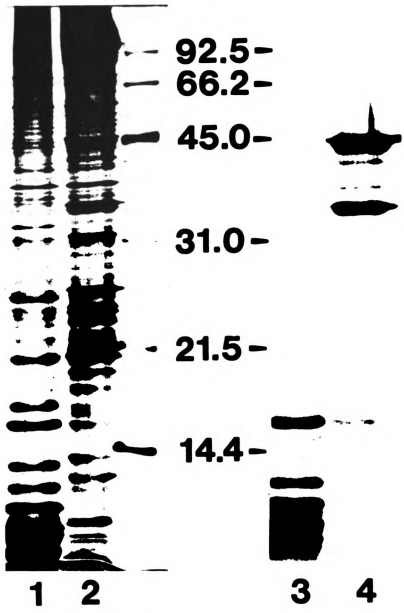

FIGURE 4. Separation of total extracellular peptides produced by Lcr^+,Pst^+ cells (lanes 1,3) and Lcr^+,Pst^- cells (lanes 2,4) of Yersinia pestis KIM; silver-stained gel (lanes 1,2) and autoradiograms of same gel (lanes 3,4).

be directed against antigens shared by Lcr$^-$ mutants thereby necessitating prior absorption with the latter. This process, of course, will remove antibodies directed against shared proteins also involved in mediating the low calcium response.

In an effort to identify all proteins synthesized at high titer during the low calcium response, we pulsed restricted cells of Y. pestis with ^{35}S-methionine and then chased the culture with the unlabeled amino acid. Although this approach was successful, it possesses certain drawbacks that require use of appropriate isolates and cultural conditions plus caution in interpretation of results. For example, in order to achieve a sufficient specific activity of incorporated ^{35}S-methionine, it was necessary to cultivate the organisms in a medium that lacked exogenous unlabeled methionine. Since Y. pestis requires this amino acid as a growth factor (3), it became necessary to first select a meiotrophic mutant capable of endogenous methionine synthesis (31). Thereafter, the organisms had to be completely restricted with no significant net increase in cell mass thereby preventing labeling of peptides normally associated with vegetative growth. For this reason, the method is only applicable to Lcr$^+$ cells of Y. pestis (because Lcr$^-$ mutants of all yersiniae and Lcr$^+$ isolates of the enteropathogenic species fail to undergo full restriction of growth and will thus incorporate radioactivity into bulk protein). Accordingly, it is not possible to perform control determinations with Lcr$^-$ organisms in order to obtain assurance that labeled peptides are indeed mediated by the Lcr plasmid. As a consequence, it became necessary to demonstrate that the molecular weights of the observed radioactive peptides corresponded to

those previously defined by other methods as being associated with expression of the low calcium response.

Evidence indicating that the seven peptides that underwent synthesis with subsequent post-translational degradation in Lcr^+,Pst^+ yersiniae were Yops is based on corresponding known molecular weights (Table 1), anatomical location within the outer membrane (Fig. 3), and stability in Lcr^+,Pst^- mutants (Fig. 2). The labeled Lcr^+,Pst^+-specific p24 was probably not a primary gene product as judged by its unusual expression. Its subsequent rapid degradation prevented localization within a subcellular fraction but an origin within the outer membrane, possibly as a degradation product of a Yop, is not unlikely. Certain evident Yops of Lcr^+,Pst^- cells of Y. pestis were excreted into the culture supernatant fluid in a manner analogous to those described for these structures in enteropathogenic yersiniae (32). Curiously, the concentrations of labeled peptides within the outer membrane of Lcr^+,Pst^- yersiniae were generally equivalent whereas those of putative excreted Yops, especially p47 and p34, were high as opposed to the remainder.

The four labeled stable cytoplasmic peptides are of considerable interest because they were expressed at evident equivalent concentrations by both Lcr^+,Pst^+ and Lcr^+,Pst^- yersiniae. One such peptide, p38, is of the same molecular weight as V antigen and monospecific anti-V sera reacted with this structure on immunoblots (not illustrated). The remaining three peptides do not correspond to any previously described Lcr plasmid-mediated structure although the possibility remains that one may represent the protein component of W

antigen. As noted above, incorporation of radioactivity into these structures does not necessarily indicate that they are encoded via the Lcr plasmid. Susequent study will be required to determine is they are unique to Lcr^+ yersiniae and, if so, are they expressed during vegetative growth or only during the low calcium response.

Further work will also be necessary to identify the Pst plasmid-mediated product that catalyzes degradation of Yops. It seems unlikely that pesticin *per se* promotes this modification because the hydrolytic specificity of this enzyme is limited to peptidoglycan (22). A more plausible explanation is that proteolysis of Yops is caused by the Pst plasmid-mediated plasmin activator. In either event, the correlation between degradation of Yops and carriage of the Pst plasmid provides an explanation for full expression of these structures in only the typically Pst^- enteropathogenic yersiniae. This relationship also clarifies why cells of *Y. pseudotuberculosis* transformed with the Lcr plasmid of *Y. pestis* are capable of maximum net production of Yops (10,33). Possession of the Pst plasmid was also correlated with *in vitro* degradation of V antigen (25). In this case, the antigen was stable in extracts of Lcr^+,Pst^- cells of *Y. pestis* and in comparable preparations of the normally Pst^-enteropathogenic yersiniae. Degradation of V in Lcr^+,Pst^+ cells of *Y. pestis* occured only after the organisms had been disrupted by sonication thus providing further evidence for a proteolytic activity compartmentalized on the outer membrane.

Further study will be required to define the biological significance of the post-translational events defined above. A simple

hypothesis is that Yops as virulence factors in Y. pestis are superseded by Pst plasmid-mediated proteolytic activity which has been associated with ability to promote disease via peripheral routes of infection (34). However, mutants of Y. pestis lacking certain Yops are avirulent (10,33) indicating that the ability to produce at least reduced levles of thes structures is also required for pathogenesis. A major conclusion of a recent review (19) was that soluble Lcr^+-specific putative virulence factors of yersiniae may also be of significance in causing disease. The results reported here provide a basis for further study of these four peptides.

MATERIALS AND METHODS

Bacteria. Y. pestis KIM was used in all experiments; this isolate is Lcr^+,Pst^+ but nonpigmented (35) and thus virulent in mice by the intraveous route but not by peripheral routes of injection (3,6) Lcr^- mutants were isolated by selection on magnesium oxalate agar (37) and Pst^-isolates were obtained by cure of the Pst plasmid by growth in the cold (25). Methionine meiotrophs (31) were isolated as described below.

Media and cultivation. Bacteria were cultivated as previously described (25) with the following minor modifications. The organisms were aerated at 26°C in the chemically defined medium of Zahorchak and Brubaker (8) to yield and optical density at 620 nm of 2. These cultures were used to inoculate second transfers of the same medium lacking L-methionine at optical densities of 0.1. These third cultures

were grown at 26°C to an optical density of 0.25 at which time they were shifted to 37°C in order to induce the low calcium response. All media contained 20 mM Mg^{2+} and no added Ca^{2+}.

Selection of methionine meiotrophs. Lcr^+,Pst^+ and Lcr^+,Pst^- organism were cultivated for two transfers at 26°C, sedimented by centrifugation at 10,000 x g at 5°C, and suspended in 0.033 M potassium phosphate buffer, pH 7.0 to an optical denstiy of about 10 ($\tilde{}10^{10}$ bacteria per ml). Samples of 0.1 ml of this suspension were spread on the surface of the methionine-free chemically defined medium containing 4.0 mM $CaCl_2$ and solidified with 1.5% agar. The bacteria were incubated at 26°C for 4 to 7 days and then individual colonies were subcultured on the ame medium. Each isolate was then assayed for ability to grow on methionine-free chemically defined medium at a typical doubling time of 2 hrs and for appropriate Lcr and Pst phenotypes. Appropriate meiotrophs of each parent strain were then used for incorporation of ^{35}S-methionine.

Pulse-chase labeling of bacteria. Bacteria were grown for 6 hrs after shift to restrictive conditions (37°C without added Ca^{2+}) and the carrier-free ^{35}S-methionine (New England Nuclear, Boston, MA, U.S.A.) was added to the cultures at a final concentration of o.1 mM). Samples of 1 ml were removed at intervals and mixed with an equal volume of cold 10% trichloroacetic acid. Precipitated material was pelleted by centrifugation at 10,000 x g for 5 minutes, washed twice in cold 5% trichloroacetic acid, and then washed twice in ethanol-ether (1:1, vol/vol). After excess ethanol-ether was volatilized at 37°C overnight, the pellets were dissolved in a minimal volume of sodim dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 2 minutes; insoluble debris was removed by centrifugation at 10,000 x g for 5 minutes. In order to facilitate autoradiography, radioactivity in a small portion of each sample was determined in in a liquid scintillation spectrometer.

Constant incorporation of radioactivity. Bacteria were labeled as described above except that ^{35}S-methionine (20 μCi/ml) was added in the presence of 2.0μM unlabeled methionine and the cultures received no excess methionine. Precipitations with trichloroacetic acid were performed as described above.

Subcellular fractionation of labeled yersiniae. Bacteria were grown in 100 ml of methionine-free medium, restricted for 6 hours, and then pulsed with 2.5 μCi/ml of carrier-free ^{35}S-methionine for 5 minutes. Unlabeled methionine (0.1 mM) was added and, after further incubation for 1 hour, the organisms were harvested by centrifugation (10,000 x g for 10 minutes) and the resulting supernatant fluid was saved. The pelleted yersiniae were washed once in 50 ml of 0.033 M potassium phosphate buffer, pH 7.0 and then fractionated by a modification of the procedure of Osborn and Munson (38) as previously modified (16) into cytoplasmic and outer membrane fractions. Portions of these samples were assayed for protein (39) and incorporated radioactivity to facilitate SDS-PAGE and autoradiography.

Autoradiography. Peptides were separated by SDS-PAGE according to the method of Laemmli (40) and gels were stained with either Coomassie Brilliant Blue G or silver stained by the method of Morrissey (41). After staining, the gels were dried and exposed to X-Omat film

(Eastman Kodak, Rochester, NY, U.S.A.) at room temperature; the autoradioagrams were aligned with stained gels to determine molecular weights of labeled peptides.

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